STUDIES ON THE ELASMOBRANCH KIDNEY. II. REABSORPTION OF UREA BY THE SMOOTH DOGFISH, MUSTELUS CANIS

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There has been evidence of reabsorption of urea by the elasmobranch tubules for over forty years. The fact that the blood and tissues contain an unusually high concentration of urea has been known since 1859, and the discovery that the urine contains a much lower level of this material was made as early as 1906. There is, therefore, a wide variation in the way in which the various kidneys treat urea: in the mammal, a back diffusion, perhaps with other factors involved (Shannon, 1936); in the aglomerular fish, probably an inward diffusion (Marshall, 1930); in the frog, secretion (Marshall, 1932; Walker and Hudson, 1937); and in the elasmobranchs, active reabsorption.

There appears to have been no adequate inquiry concerning the detailed behavior of the elasmobranch kidney in relation to urea, despite the long existence of evidence of its reabsorption and its quantitative demonstration by Clark and Smith (1932).

There has been no demonstration of the site of urea reabsorption. It was suggested by Marshall (1930) and by Smith (1931) that the "special segment" of the elasmobranch tubule is responsible for urea reabsorption. This has been accepted by Baldwin (1937) and other writers. However it has been shown by direct puncture methods that this special segment does not exist in the tubule of the spiny dogfish, *Squalus acanthias* (Kempton, 1943). Moreover there is reason to believe that the concept of a special segment is a mistake and that the segment does not exist in any elasmobranch tubule.

Attention now has been turned to a study of urea reabsorption by the entire kidney with the hope that such an investigation might give some clue concerning the reabsorptive mechanism. This primary objective was successful to the extent that some of the factors controlling the rate of urea reabsorption have been revealed. Conclusive evidence as to the site of reabsorption in relation to other tubule functions has not been found.

Materials and Methods

The simultaneous reabsorption of water, urea and glucose has been studied, using inulin as a measure of glomerular filtration. Clearances were calculated by use of the well-known method of Smith (1939): UV/P, in which U is the mgm.% of the material in the urine, V is the volume of urine in cc./hr./k., and P is the mgm.% present in the plasma. Contrary to the usual practice in urea studies, plasma concentrations instead of whole blood were used because of the finding by Dr. Arthur K. Parpart (personal communication) that the erythrocytes of this species are only slowly permeable to urea.

For inulin determinations the resorcinol method of Hubbard and Loomis (1942) was employed, with two modifications. Since it was more convenient to use larger cuvettes in the spectrophotometer, 2.0 cc. samples of the plasma or urine filtrate diluted to the range of the method were used, with the final dilution in sugar tubes to the 12.5 cc. level. Heating was extended from 8 to 16 minutes, a change which produced somewhat more constant results. The tubes were read against a blank of the reagents in distilled water. Use of other blanks recommended by Hubbard and Loomis was tried repeatedly, but since no effect was ever found their use was dropped in the routine analyses. The method as modified was found to be very satisfactory, and it was not affected even by elevated glucose levels. Special tests indicated that interference from this source did not appear until the glucose level in the diluted plasma or urine filtrate was raised to about ten times the concentration of inulin.

The urea method was a slight modification of the urease and direct Nesslerization method of Koch and Hanke (1948). Because this method was developed for the relatively low plasma level and the high urine level of mammals, several changes had to be introduced to care for the reversed situation found in the elasmobranchs. Plasma was first diluted to range (usually a 20 × dilution) and then digested with Koch's glycerol urease extract at 50° C. for 60 minutes. In preparing the filtrate, the sodium tungstate and sulfuric acid were reduced in amount to 0.5 cc. each, and the normality of the acid was raised to 1.0. After filtration and Nesslerization, the readings were made against ammonium sulfate standards. Urine was treated by the same method, except that the initial dilution was usually 6 ×. The method as used includes any ammonia nitrogen which might be present. It was deemed inadvisable to attempt to separate the two sources of nitrogen because there might be some urea decomposition during the prolonged collection periods, and in addition Denis (1922) found very little ammonia nitrogen in elasmobranchs.

The method as modified was satisfactory for the purpose, but it had two objectionable features: the high dilution magnified very greatly any differences in the spectrophotometric readings; for some reason a blank containing urease gave an undue amount of light scatter. In a few cases the urine samples also gave excess scatter and had to be discarded. The source of this effect was not determined.

For glucose analyses the arsenomolybdate method of Nelson (1944) was selected. Undiluted plasma filtrates and diluted (4 ×) unfiltered urine were used. The blank consisted of the normal reagents in distilled water.

All readings were made with a Coleman Junior Spectrophotometer with cuvettes 19 mm. in diameter. Wave-lengths used were as follows: inulin method, 495 millimicra; urea, 560; glucose, 500.

Only female dogfish were used. This was due partly to the small number of males actually caught, and partly to possible complications resulting from the use of the same ducts for seminal fluid and urine. The weight of the experimental fish ranged from 5.3 to 10.3 kilograms. Below this range the urinary papilla was so small that urine collections were impracticable; at the top weight the animals were so strong that it was barely possible to handle them without damage to the balloon which collected the urine. During May and June animals of the necessary size were obtained from the traps of commercial fishermen, but after about the first of July it was necessary to catch them by hook after sunset either in deep water by the shore or from a boat in the deeper water of the harbor. The fish were always

transferred immediately to a tank of fresh sea water and rushed to a live car in a float, by which tidal currents swept at a rate sufficient to ensure rapid and complete change of water (or, in 1950, to a large concrete tank with well aerated running water). The animals refused even living food in captivity; in fact they were very likely to regurgitate food which had been taken before capture. In a few cases the hook wound continued to bleed, especially when the animals were handled for collecting blood and urine. (These animals were discarded.) At best the life in captivity was limited to a few days, and there was some evidence that this was in part a temperature effect. As the water temperature became higher during the summer their life expectancy became reduced. The warmer waters of the Eel Pond in mid-summer killed dogfish in a few hours. A period of exceptionally hot weather had a very deleterious effect on the animals in use at the time. The more constant temperature in the concrete tank allowed the animals to remain in good condition for several days longer than when the live car was used. Usually the experiment was ended when the urinary papilla became so damaged that it would no longer support a catheter. The animals usually lived several days after the enforced termination of the collections. It was only in the most prolonged experiments that there was any appreciable reduction in the blood sugar level.

The animals were prepared for the collection of urine by the removal of the tip of the urinary papilla, and the insertion into the papilla of an in-lying catheter of glass. A rubber balloon, tied securely to glass tubing, was anchored by ligature to the ventral surface of the tail. The catheter was connected with the glass tubing by a piece of flexible rubber tubing, with sufficient slack to permit swimming and movements of the cloacal region without placing a strain on the catheter and the papilla. At the end of each collection period, the ligature holding the balloon was cut, the tubing disconnected from the catheter, and the urine poured into a collecting vessel. The abdomen was massaged carefully to force from the kidney ducts any fluid they might contain, this urine being removed from the catheter by syringe. The amount obtained by massage usually varied from zero to five cubic centimeters, although in one very exceptional case 40 cc. was collected. The balloon was then replaced by a fresh one. These balloons were of sufficient size to ensure that no back-pressure developed. These and other manipulations were performed on the float containing the live car, or beside the concrete tank. This made it possible to transfer the animal quickly from the circulating water to a trough, where it was tied on its back with its head immersed in fresh sea water. No anesthetic was ever used.

Administration of various materials was by diverse routes. Inulin, prepared as a 20% solution in warm distilled water, was injected intravenously through the caudal blood vessel. In some experiments other materials were also injected in the same way. Water was sometimes given by stomach tube, but this was not the standard procedure. Urea was administered in three different ways. Intravenous injections were fatal quickly. In a number of cases intraperitoneal injections were also fatal, but more slowly, with the liver exhibiting severe lesions. Intramuscular injections were partially successful, since an increase of plasma urea levels ranging up to 50% was obtained in about half of the injected animals. The firm unyielding nature of the flesh made difficult the injection of more than a few cubic centimeters at any one point. It was necessary therefore to make a series of small injections along the epaxial muscles of both sides. The dosage was 10 cc. per kilogram of a

50% solution in distilled water, freshly prepared at room temperature. In some cases this dosage was repeated twice, giving a total urea administration of 15 grams of urea per kilogram of body weight.

Blood was collected by syringe in amounts of 10 cc., and was transferred at once to test tubes containing 0.1 mgm. potassium oxalate, precipitated by drying from a

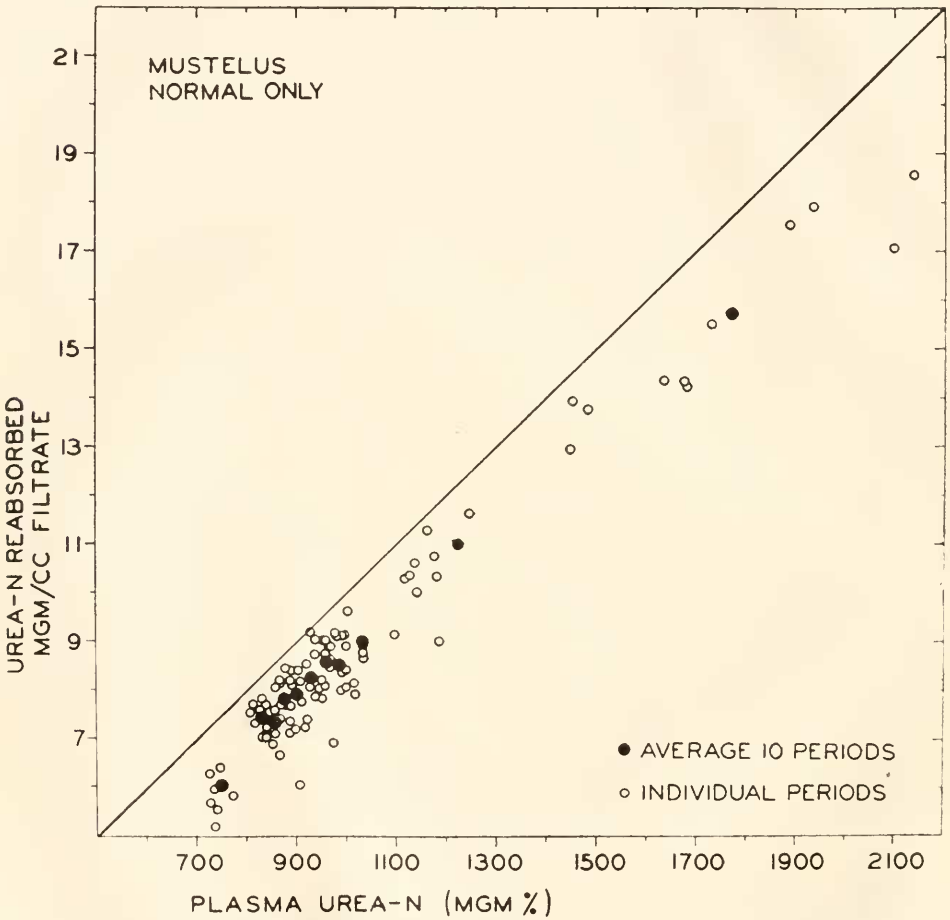

FIGURE 1. Relationship between the reabsorption of urea from each cubic centimeter of filtrate and the plasma urea level; 110 collection periods from smooth dogfish in good condition. The abscissa could be labelled equally well "Filtrate urea-N (mgm.%)" instead of referring to plasma level. For each point, the vertical distance to the diagonal line represents the unreabsorbed moiety; the vertical distance extrapolated to a zero baseline represents the reabsorbed urea.

20% solution. Upon return to the laboratory from the float or tank this oxalated blood was centrifuged immediately, the plasma either being used at once or being stored over night in a very cold refrigerator. When haematocrit readings were made, capillary tubing of uniform internal diameter of 0.6 mm. was used, the centrifugation being for one-half hour in an International clinical centrifuge using

direct current. Top speed of 3000 r.p.m. gave a centrifugal force of 1600 × gravity. This force, for this period of time, gives as complete packing as an air turbine developing 20,000 × gravity (according to turbine determinations by Dr. James W. Green).

The routine plan of the experiments, from which there was considerable deviation, called for the capture of the fish one evening, its weighing the next morning, the injection of inulin early that afternoon, and the start of the first collecting period the next morning. Collections of blood were made at the beginning and end of each period. Urine was collected at the end of the period. Usually collections were made morning, early afternoon and evening, giving periods of approximately 12, 6, and 6 hours. In many cases a midnight collection was made also, thus dividing the day approximately into four six-hour periods. The delay between the injection of the inulin and the start of the first collection period placed all periods on a fairly flat portion of the inulin tolerance curve.

Results

There is a linear relationship between the reabsorption of urea from each cubic centimeter of filtrate and the plasma urea concentration (or the filtrate concentration). In general, regardless of the concentration of urea in the plasma and filtrate, all of the urea is reabsorbed except a constant residuum which stays in the tubule. This relationship is true on the average (Fig. 1), the minor variations in the unabsorbed urea being related to other factors as indicated below. This general relationship holds true over a range of normally occurring plasma levels in which the highest is approximately triple the lowest. Of all the data obtained, this average level of the unabsorbed urea in the tubules is the only constant factor. Raising the urea level, in an attempt to determine whether there is a definite tubular maximum at which the relationship would no longer hold, met with failure. Fortunately the range of normal variation of plasma level was sufficiently great to permit the linear relation to appear.

From *a priori* considerations there are a number of other correlations which might be expected. Surprisingly these do not appear and some of these negative results seem to have interpretative significance. Factors which show no correlation include the following combinations:

(1) Plasma urea level (mgm.%) and urea reabsorption (mgm./hr./k.)
(2) Plasma urea level (mgm.%) and urea filtered (mgm./hr./k.)
(3) Filtration rate (cc./hr./k.) and urea reabsorption (mgm./hr./k.)
(4) Filtration rate (cc./hr./k.) and urea reabsorption (mgm./cc. filtrate)
(5) Urea reabsorption (mgm./hr./k.) and glucose reabsorption (mgm./hr./k.)
(6) Inulin U/P ratio and urea U/P ratio
(7) Urea filtered (mgm./hr./k.) and urea reabsorbed (mgm./hr./k.)
(8) Urea filtered (mgm./hr./k.) and urea reabsorbed (mgm./cc. filtrate)
(9) Plasma urea level (mgm.%) and % urea reabsorbed
(10) Glucose reabsorbed (mgm./hr./k.) and urea reabsorbed (mgm./cc. filtrate)
(11) Urine urea level (mgm.%) and urea reabsorbed (mgm./cc. filtrate)

There is no reason to believe that the lack of correlation is due to incomplete emptying of the kidney ducts, although this is theoretically possible in some cases.

In the first place, every effort was made to massage all the urine from the ducts. Secondly, when the data are in terms of a unit volume of filtrate, as in 4, 8, 10 and 11 above, uncollected urine could have only a very slight effect on the calculations.

The scatter in the data is not merely fortuitous. While the main amount (average 87%) of urea reabsorption from each cubic centimeter of filtrate is correlated with the plasma (and filtrate) level, the variations in unabsorbed urea are correlated with water reabsorption (Fig. 2), urine flow (Fig. 3), and urine urea concentration (Fig. 4). In other words, the variations of unabsorbed urea are correlated with the behavior of the tubules toward water, at least in part, with more

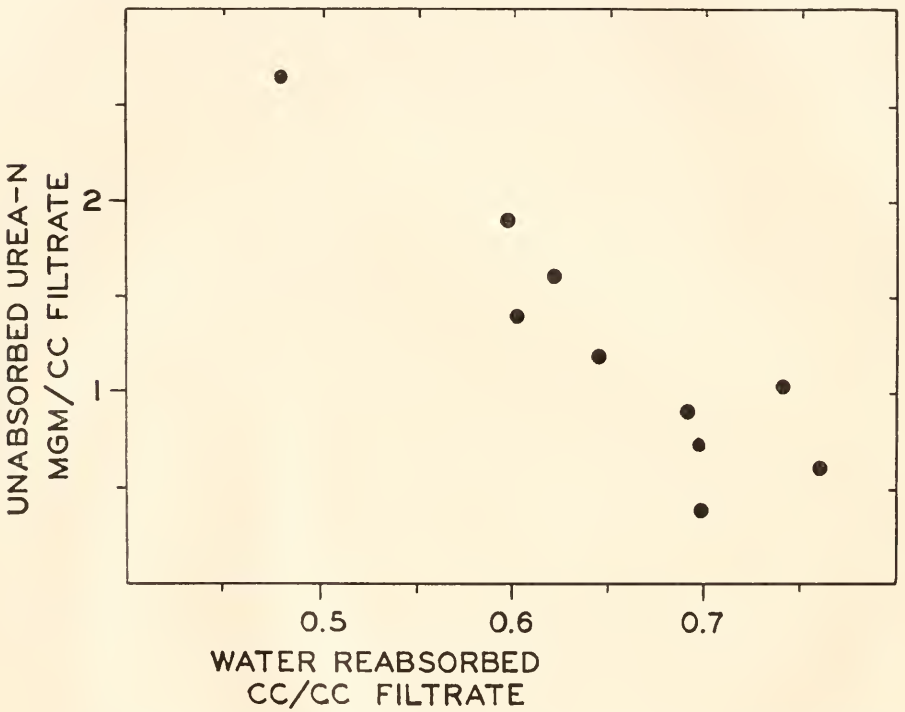

FIGURE 2. Variation in the unabsorbed urea in relation to the amount of water reabsorbed, both in terms of each cubic centimeter of filtrate. Each point represents the average of 10 collection periods.

urea remaining in the tubules unabsorbed when there is less water reabsorption, a higher urine rate, and concomitantly a higher concentration of urea in the urine. It should be pointed out that these variations in unabsorbed urea are definitely not correlated with differences in the rate of filtration, of glucose reabsorption, or the absolute rate of water or urea reabsorption. This will be referred to in the discussion.

As mentioned previously, an attempt was made to raise the urea level of the blood above the normal range. This proved to be difficult. One great obstacle was the fact that following urea injections the animals tended to continue to bleed

at every point of urea injection, and even to start bleeding at the points from which blood had been withdrawn previously. Even in the most successful experiments, the haematocrit reading fell drastically, and the blood became extremely fluid. How much of this was due to continuous seepage of blood outward, and how much, if any, was due to an inward diffusion of water through the gills, remains obscure.

The greatest increase in plasma urea-N level obtained by urea injections was from 870 mgm.% before the injection to 1230 mgm.% afterward. The highest level obtained was 1550 mgm.%, a rise from a pre-injection level of 1240 mgm.%. These new levels were well below the maximum found occurring naturally. In

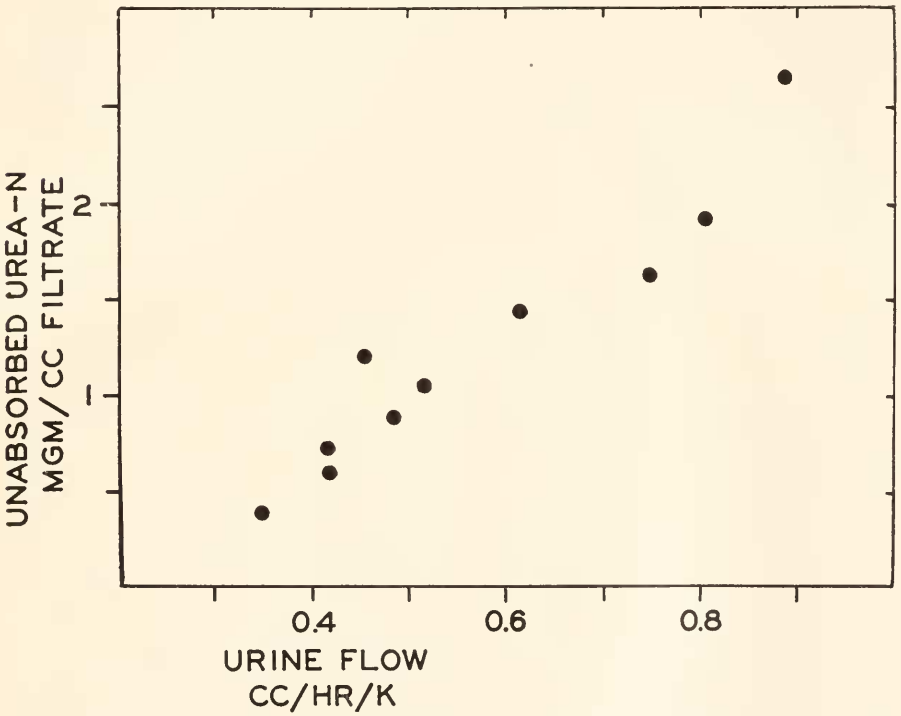

FIGURE 3. Variation in the unabsorbed urea in relation to the rate of urine flow. Each point represents the average of 10 collection periods.

these urea-injected animals there was no demonstration of a definite tubular maximum, but instead the reabsorption of urea from each cubic centimeter of filtrate was markedly depressed as compared with the uninjected animals. In preliminary results reported earlier (Kempton, 1948) the rate of reabsorption after injection was of the order of magnitude to be expected at the urea level prevailing before the injection. However, more data indicate that this was fortuitous, the rate of urea reabsorption not being "set" in relation to the pre-injection urea level.

This depression of urea reabsorption is probably without significance in relation to the reabsorptive process, because glucose and water reabsorption were similarly depressed. A comparison is given in Table I, in which the averages of the urea

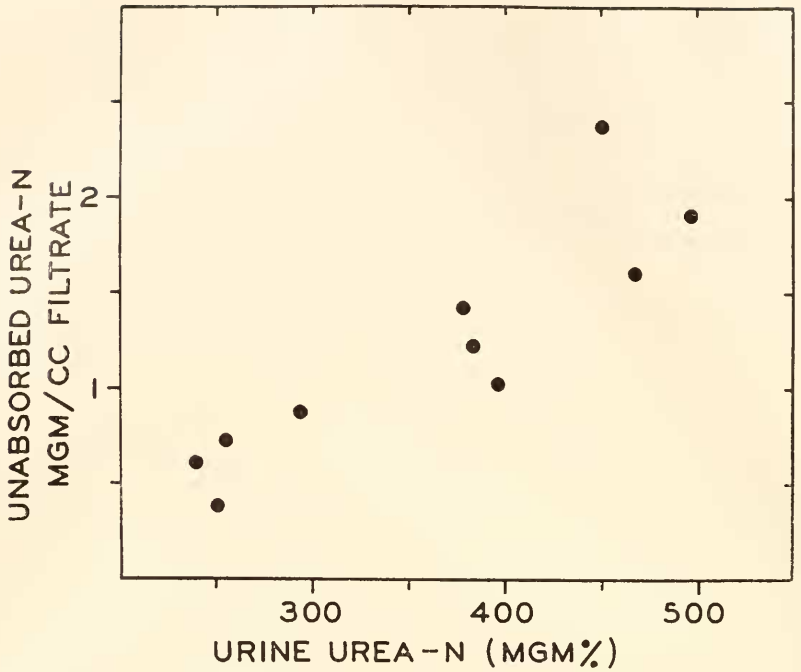

FIGURE 4. Variation in the unabsorbed urea in relation to the concentration of urea nitrogen in the urine. Each point represents the average of 10 collection periods.

TABLE I

Comparison of averages of urea-injected animals with normal ones at the pre-injection and post-injection levels

	I	II	III
Plasma urea-N, mgm. %	898	1196	1122
Urine urea-N, mgm. %	396	1083	306
Filtration rate, cc./hr./k.	2.38	1.64	1.68
Urine rate, cc./hr./k.	0.66	0.83	0.69
H_2O reabsorbed, cc./hr./k.	1.69	0.80	1.00
Urea-N reabsorbed, mgm./hr./k.	17.6	10.6	16.6
Glucose reabsorbed, mgm./hr./k.	4.5	2.1	3.4
Reabsorption per cc. of filtrate:			
H_2O (cc.)	0.72	0.47	0.53
Urea-N (mgm.)	7.76	6.04	9.75
Glucose (mgm.)	2.01	1.31	2.22

Column I. Group of 10 normal animals closest to the average plasma level of the injected animals previous to their injection.

Column II. Average of 9 collection periods from 5 animals injected with copious amounts of urea.

Column III. Group of 5 normal animals nearest to the average plasma level attained after the urea injections were made. More animals could not be used in this group because of the smaller numbers available at this relatively high urea level.

injected animals are compared on the one hand with an uninjected group selected to have a plasma level close to those prevailing before the urea injections were made, and on the other hand with a group of animals whose average plasma level compared with the experimental animals after the massive urea injections. If the kidney were "set" in its behavior by the pre-injection level, the second column of the table should correspond with the first; if the kidney behaved toward the injected urea as it does toward the endogenous urea there should be a correspondence between the second and third columns. Actually neither relation appears, but there is indication of a general depression of renal function as shown by the filtration rate, water reabsorption, urea reabsorption and glucose reabsorption.

TABLE II

Data from 110 collection periods from smooth dogfish in good physical condition which had received inulin injections but no urea, based on data of summers of 1948 and 1950

	Minimum	Average	Maximum
Urine flow, cc./hr./k.	0.11	0.63	1.72
Filtration rate, cc./hr./k.	0.53	1.82	3.51
Urea clearance, cc./hr./k.	0.05	0.24	0.81
Glucose clearance*, cc./hr./k.	0.03	0.16	0.29
Inulin U/P ratio	1.10	3.28	7.07
Urea U/P ratio	0.13	0.39	0.74
Plasma urea-N, mgm. %	730	1014	2140
Urine urea-N, mgm. %	120	373	603
Plasma glucose,* mgm. %	129	253	529
Urine glucose,* mgm. %	13	54	112
Per cent reabsorption:			
H_2O	9	66	86
Urea-N	70	87	99.5
Glucose*	86	92	99.0
Reabsorption per cc. of filtrate:			
H_2O (cc.)	0.09	0.65	0.86
Urea-N (mgm.)	5.2	8.9	18.5
Glucose (mgm.)*	1.2	2.3	3.5

* For glucose, only 51 collection periods are represented.

Since there is a paucity of data in the literature, it seems worthwhile to present some of the routine values obtained in these experiments, even though their significance for the present study is incidental. These are summarized in Table II. The values were obtained from animals which were used for a total of 110 collection periods during the summers of 1948 and 1950. There is no available explanation for the fact that the rates of urine flow obtained in a very short series of experiments in the summer of 1947 (Kempton and Steckler, 1947) were of a different order of magnitude, reaching a maximum of 3.94 cc./hr./k. as contrasted to 1.72 in the other two summers. In other respects these earlier animals were entirely in accord with the findings in 1948 and 1950, but because of the extreme differences in urine flow, and the possibility that this was a technical artifact due to unskillful handling of the animals, the few 1947 data have been omitted. Also omitted are the animals to which urea was administered, and a few periods in which

the fish appeared to be moribund. Twelve collection periods with one spiny dogfish, *Squalus acanthias,* in 1948 gave values which were entirely in line with those of the smooth dogfish.

Discussion

The data indicate that the rate of urea reabsorption is geared, directly or indirectly, to the plasma urea level. The only constant value is the average amount of urea remaining in the tubules unabsorbed from each cubic centimeter of filtrate. This relationship prevails through a range of normally occurring plasma levels in which the maximum is triple the minimum.

The reason that a similar relationship does not appear between total urea reabsorption in terms of mgm./hr./k. and the plasma concentration seems to be clear. In such a calculation the amount of urea would depend upon both the concentration in the filtrate and the volume of the filtrate. As the latter changed so would the amount of urea reabsorbed. But since the reabsorption is geared to the amount remaining unabsorbed in each unit volume of filtrate, the variations in total filtration introduce a random element which eliminates any apparent relationship.

The rate of reabsorption from each cubic centimeter of filtrate is not altered appreciably by the amount of filtrate produced nor the total amount of urea filtered. If all glomeruli are functioning all the time, differences in rate of filtration should result in a different rate of flow along the tubule, which in turn should be mirrored in variations in rate of urea reabsorption. This effect should certainly be clear when, as in these data, the rate of filtration falls along a seven-fold range. However, changes in filtration rate due to varying numbers of functional glomeruli would not result in a change of rate of flow. The fact that there is no change in urea reabsorption which can be correlated with changes in filtration rate indicates that the varying filtration rate in these experiments was produced by a change in the number of functional glomeruli rather than by increased or decreased function of individual units.

The variations in unabsorbed urea can be explained on either of two bases. These lead to two diametrically opposed conclusions in relation to the site of urea reabsorption as compared with that of water reabsorption. Variations in rate of filtration do not affect the urea reabsorption, apparently because there is no change in rate of flow of the filtrate, which has been discussed above. However, changes in amount of water reabsorption are related inversely to the amount of unabsorbed urea. This would be consistent with the idea that the osmotic effect of the greater amount of urea retained in the tubule inhibits reabsorption of water and leads to greater urine flow and a higher concentration of urea in the urine. The facts equally well fit the explanation that with less reabsorption of water there is a more rapid flow of filtrate which results in increased amounts of unabsorbed urea and higher urine levels. The first explanation would place urea reabsorption earlier in the tubule than water reabsorption or at the same level; the second would place it after the site of water reabsorption. No choice between the alternatives can be made on the basis of the present data. It is hoped that further experiments will permit a definite choice.

The reabsorption of urea cannot be isosmotic. While at lower plasma levels enough water is reabsorbed to account for an isosmotic reabsorption of all the urea

and possibly the glucose and chloride as well, at higher plasma levels only a small fraction of the urea could be absorbed as an isosmotic solution, even if all the reabsorbed water were assigned to this purpose. This therefore leaves open the possibility that the controlling factor in urea reabsorption is a constant concentration of urea remaining in the tubules, possibly through the intervention of osmotic relationships. It is perhaps more likely that we are dealing with an enzyme system which functions rapidly only when a certain concentration of urea is present in the tubules.

Summary and Conclusions

1. Water, urea and glucose reabsorption have been studied simultaneously using the inulin clearance method.

2. Variations in filtration rate appear to be due to changes in the number of functional glomeruli, rather than to changes in the rate of function of individual units.

3. Glucose reabsorption is not correlated in any way with that of urea.

4. The main factor controlling the urea reabsorption is the concentration of urea occurring normally in the plasma. Attempts to increase the reabsorption of urea by raising the plasma level result in a depression of reabsorption not only of urea but of glucose and water as well. To some extent this reduced water reabsorption is offset by a decreased filtration rate, with the result that only a very moderate diuresis ensues.

5. The percentage of the filtered urea which is reabsorbed varies with the concentration in the plasma, ranging from 70% to 99.5%, comparing quite favorably with the reabsorption of glucose.

6. On the average, the actual amount of unabsorbed urea left in each unit volume of filtrate is quite constant over a wide range of normally occurring urea levels.

7. Variations in the amount of unabsorbed urea are correlated in a presumably causal fashion with the reabsorption of water, the rate of urine flow and the concentration of urea in the urine.

8. Choice cannot be made at this time between alternate explanations of the data which would localize the site of urea reabsorption in relation to the site of water reabsorption.

9. The data show clearly that the reabsorption of urea cannot be an isosmotic one.

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